TGF-β and αvβ6 integrin act in a common pathway to suppress pancreatic cancer progression

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Abstract

The TGFβ pathway is under active consideration as a cancer drug target based on its capacity to promote cancer cell invasion and to create a pro-tumorigenic microenvironment. However, the clinical application of TGFβ inhibitors remains uncertain as genetic studies demonstrate a tumor suppressor function of TGFβ in pancreatic cancer and other epithelial malignancies. Here, we used genetically engineered mouse models to investigate the therapeutic impact of global TGFβ inhibition in pancreatic cancer in relation to tumor stage, genetic profile, and concurrent chemotherapy. We found that αvβ6 integrin acted as a key upstream activator of TGFβ in evolving pancreatic cancers. In addition, TGFβ or αvβ6 blockade increased tumor cell proliferation and accelerated both early and later disease stages. These effects were dependent on the presence of Smad4, a central mediator of TGFβ signaling. Therefore, our findings indicate that αvβ6 and TGFβ act in a common tumor suppressor pathway whose pharmacologic inactivation promotes pancreatic cancer progression.

Introduction

The transforming growth factor-β (TGFβ) signaling pathway is an evolutionarily conserved regulator of embryonic patterning and cell differentiation, and has central roles in wound healing and inflammation (1). The activated TGFβ receptor (TGFβR1/R2) phosphorylates the Smad2 and Smad3 proteins, which modulate transcription in association with Smad4. This pathway has been the subject of intense investigation in cancer due to its potential to act in both a pro- and anti-tumorigenic manner (2). Depending on cross-talk with other pathways, TGFβ can inhibit proliferation and suppress transformation by modulating expression of cell cycle regulators. Alternatively
TGFβ can promote malignant growth through multiple mechanisms including enhanced cancer cell invasion, survival, matrix remodeling, fibrosis, and immunosuppression.

As in other epithelial cancers, TGFβ pathway function in pancreatic ductal adenocarcinoma (PDAC) appears complex. Inactivating mutations in SMAD4 and other pathway components are present in ~50% of human PDAC and cooperate with activated Kras$^{G12D}$ to promote PDAC in mouse models (3-6). However, TGFβ ligands are commonly over-expressed in PDAC, and can promote epithelial-to-mesenchymal transition (EMT) and invasion in cell lines (7, 8). TGFβ can also induce angiogenesis, activate tumor-promoting myofibroblasts (stellate cells), and attenuate immune surveillance (9, 10). In light of these observations, TGFβ inhibitors are under investigation as PDAC therapeutics and have shown efficacy in xenograft studies (11, 12).

The multifaceted and cell-type specific effects of TGFβ inhibition present problems in fully assessing the clinical utility of drugs against this pathway. Such effects are likely to be best-understood using native cancer models that appropriately recapitulate tumor-stroma interactions as well as the multistage progression that defines human cancers. Here, we investigated the upstream regulation of TGFβ signaling in the pancreas to establish new strategies to target the pathway, and we examined the impact of pharmacologic inactivation of multiple TGFβ signaling components using genetically engineered mouse (GEM) models of PDAC. These studies, carried out in the context of sequential tumor stages, different genetic lesions, and combined treatments with cytotoxic chemotherapies, failed to reveal a therapeutic window. Instead we found multiple settings where disease was exacerbated by TGFβ inhibition. This preclinical information does not presently support the utility of broadly targeting this pathway in PDAC.
**Materials & Methods:**

*Mouse models:* All treatment studies were conducted in accordance to UCAR and institutional standards using previously described mouse strains (5). Littermates were distributed among 1D11 (anti-Tgfβ), 13C4 (IgG isotype control), and 3G9 (anti-αvβ6) groups (13, 14). Gemcitabine was dosed at 100mg/kg IP twice weekly. Mice were treated at age six weeks and euthanized at 12 weeks (PanIN study) or at nine weeks until exhibiting signs of illness (PDAC study). In the PDAC cohort four long-lived controls were sacrificed and censored after 20 weeks of age when all mice in the experimental cohorts had died. These animals were free of signs of illness but upon pathologic evaluation were found to have advanced PanIN or early cancers.

*Histological analysis:* PanIN/PDAC tumor burden was determined by serial analysis of >3 H&E sections through the longitudinal plain of the pancreas. A gastrointestinal pathologist (V.D.) determined percentage of pancreas occupied by normal tissue, PanIN and PDAC, in a blinded fashion.

Antibodies: for αvβ6, the mAb 6.2A1 (14) used at 1:100 in human tissue or the human/mouse chimeric form of 6.2A1 (ch6.2A1) in mouse tissue (15) used at 1:100; for phospho (Ser465/467)-Smad2, Cat#AB3849 (Millipore Corporation); for endothelial cells, the rat endomucin v.7C7 (Santa Cruz) used at 1:50; for pericytes, NG2 Cat# AB5320 (Chemicon) used at 1:200; for Ki-67, NCL-Ki67p (Novocastra); for macrophages, the anti-CD68-M antibody, MCA1957T (Serotech); for total T-cells, the anti-CD3 antibody, Cat# RM-9107-S (Lab vision/Neomarkers); for Foxp3, Cat#14-5773 (eBioscience).

*Quantification of IHC/ IF:* Staining for CD68, FoxP3 and phospho-Smad2 was quantified by scanning slides at 20x using the Aperio-XT automated imaging system. Regions of
interest where identified within the tissues for quantification of DAB positive CD68 and Foxp3 stained cells. For phospho-SMAD2 quantification, we used an automated algorithm to quantify the level of nuclear DAB staining on a scale ranging from 0, +1, +2 and +3. Ki-67 staining was quantified by pathologic evaluation as the percent of neoplastic cells with positive staining.

**Statistical analysis:** Survival was determined using the Kaplan-Meier method and comparisons were determined using the Log-rank test. Animals showing signs of illness and with confirmed cancers were included as events, whereas animals that died for reasons other than cancer were censored. Histological scores for disease burden, Ki67, and P-smad2 staining between treatment groups compared using t-tests. β6 IHC scoring was compared by the Mann Whitney test.

**Results**

PDAC evolves from premalignant lesions including acinar-to-ductal metaplasia (ADM) and pancreatic intraepithelial neoplasia (PanIN) (16). We evaluated the activation status of the TGFβ pathway during PDAC progression via immunohistochemical staining for Serine465/467-phosphorylated Smad2 (phospho-Smad2) in the *Ptf1-Cre;LSL-KrasG12D; p53Lox/+* (*Kras-p53Lox/+*) model. Phospho-Smad2 was elevated in ADM and early PanINs compared to normal ductal and acinar cells, and remained at high levels throughout PDAC progression (Figure 1A, upper panels, yellow arrowheads). Stromal fibroblasts also showed strong nuclear P-Smad2 staining (Figure 1A, red arrowheads).

Previous studies have documented increased expression of TGFβ ligands in PDAC progression (5). Because TGFβ is produced as a latent complex, additional processes such as proteolytic cleavage or conformational changes are required to
activate signaling. To determine the basis for TGFβ activation, we examined the expression pattern of αvβ6, a candidate activator of latent TGFβ that is upregulated in advanced tumors (15, 17). In mice, αvβ6 was absent in islet and acinar cells and at low to moderate levels in normal ducts, whereas expression was increased throughout each stage of PDAC progression (Figure 1A, lower panels). Expression of αvβ6 was restricted to transformed pancreatic ductal epithelium with no evidence of staining in the stromal microenvironment. Human specimens showed a similar αvβ6 expression profile, with staining in low- and high-grade PanIN lesions and most PDAC, with normal human ducts showing only weak staining (Figure 1B). The correlation between induction of αvβ6 expression and phospho-Smad2 in PanIN and PDAC suggests this integrin may be important in local activation of TGFβ signaling in ductal lesions.

To examine the relationship between αvβ6 function and TGFβ signaling we treated Kras-p53Lox/+ mice with an αvβ6 blocking IgG monoclonal antibody (3G9) (14), a pan-TGFβ blocking IgG monoclonal antibody, 1D11 (13), or an isotype control antibody (13C4). Anti-αvβ6 treatment strongly decreased phospho-Smad2 expression in PanIN and PDAC lesions as well as surrounding stroma (Figure 1C). Collectively, our data indicate that αvβ6 is critical for activation of TGFβ signaling in the neoplastic epithelium.

Targeting TGFβ signaling could limit PDAC growth by blocking TGFβ-mediated pro-tumorigenic effects on the microenvironment and on the invasiveness of cancer cells. Moreover, αvβ6 inhibition could serve to inactivate TGFβ signaling in a restricted manner, limiting the effects of a pharmacologic blockade to the diseased pancreas. To test these possibilities we used the anti-TGFβ, anti-αvβ6, and isotype control antibodies in the Kras-p53Lox/+ model. To evaluate the impact of treatments on progression of pre-invasive lesions antibodies were administered at 5 weeks of age— when the pancreas is largely normal but contains focal early stage PanINs (schematic in Fig. 2A). Pancreases...
were evaluated for the presence of gross tumors and by correlative histological and immunohistochemical analysis at 12 weeks. Anti-\(\alpha v\beta 6\) treated animals had increases in the proportion of the pancreas exhibiting PanIN or PDAC lesions compared to controls (mean = 73\% in 3G9 vs. 45\% control; \(p=0.04\); Figure 2B, upper row), as well as a higher frequency of invasive PDAC (66\%, versus 33\% in controls). Comparable increases in neoplasia were observed in anti-Tgf\(\beta\) treated mice. Therefore, blocking \(\alpha v\beta 6\) accelerated the course of PanIN initiation and progression, leading to a larger burden of disease and more advanced tumors.

Acceleration in progression of PanIN lesions among anti-Tgf\(\beta\) and anti-\(\alpha v\beta 6\) treated mice was associated with an increase in proliferation as reflected by Ki67 staining (Figure 2B, lower row). Consistent with this we observed that 4/5 primary pancreatic ductal cell cultures with activated Kras demonstrated growth inhibition in responses to TGF\(\beta\) treatments. We failed to observe significant alterations in stromal components that can be activated by TGF\(\beta\), including the stellate cells (smooth muscle actin), T\(_{\text{regs}}\) (FoxP3), macrophages (CD68), the desmoplastic stroma (qRT-PCR analysis for collagen-1) and vasculature (endomucin and NG2) (Supplemental Figure 1A-C). Thus, these data indicate that the \(\alpha v\beta 6\)-TGF\(\beta\) pathway has a primary role in restraining proliferation and malignant progression of PanIN epithelial cells.

Since both TGF\(\beta\) and \(\alpha v\beta 6\) signaling have been implicated in the induction of EMT and invasive growth of established cancers, we next sought to test whether the anti-TGF\(\beta\) and anti-\(\alpha v\beta 6\) antibodies had a differential impact at later disease stages. \(\text{Kras-p53}^{\text{Lox/+}}\) mice were treated beginning at 9-10 weeks of age, when either high grade PanINs (PanIN-3) or locally invasive PDAC were present (schema in Fig. 2C, upper). Mice were treated until signs of illness necessitated euthanasia. Notably, overall survival was significantly diminished in the anti-TGF\(\beta\) and anti-\(\alpha v\beta 6\) groups demonstrating a
persistent role for TGFβ in suppressing growth at later stages of disease (Figure 2C, lower left). Histopathological analysis revealed treated tumors to be invasive PDAC showing a range of histological differentiation. Blocking antibodies did not produce significant differences in the spectrum of tumor grade and histological subtypes (Supplemental Figure 2A&B).

The PDAC stroma is a potential barrier to effective delivery of chemotherapeutic agents to tumor cells (18). Based on the potential function of TGFβ signaling in activating stromal fibroblasts, we tested whether TGFβ blockade influenced the response of the Kras-p53Lox/ model to gemcitabine, a standard chemotherapy. The addition of αvβ6 or TGFβ blocking antibodies to standard gemcitabine treatment led to a diminished survival as compared with gemcitabine alone (Figure 2C, bottom right). Therefore, αvβ6 and TGFβ restrain the initiation and progression of PDAC, apparently through functions on the neoplastic epithelium; potential positive roles of αvβ6 and TGFβ in stromal regulation may have a less prominent impact on tumorigenesis.

Our work shows that αvβ6 is a critical component of the TGFβ-Smad4 tumor suppressor pathway in PDAC. Correspondingly, reduced αvβ6 expression could serve as an alternative mechanism to SMAD4 mutations as a means to inactivate the TGFβ pathway during PDAC progression. To examine this question, we performed IHC analysis across a set of PDAC specimens derived from Smad4 wild type and Smad4 null mouse models using antibodies to αvβ6. While all the tumors with Smad4 mutations expressed αvβ6 at higher levels in invasive tumors compared to PanINs, we found that ~26% of Smad4 wild type tumors lost αvβ6 expression (Figure 3A). Importantly, the absence of αvβ6 staining correlated with SMAD4 status (p=0.01). The spontaneous loss of expression of αvβ6 among Smad4 wild-type tumors, but never in combination with Smad4 mutation, supports the view that αvβ6 is a central activator of the TGFβ-SMAD4
tumor suppressor pathway, and suggests that molecular alterations of both upstream and downstream components promote PDAC tumorigenesis.

To test more directly whether αvβ6 acts in a common TGFβ/Smad4 tumor suppressor pathway, we assessed the impact of 3G9 on a Kras-driven PDAC model that also has an engineered homozygous deletion of Smad4 and therefore has defective TGFβ signaling in the pancreatic epithelial cells (5). Treatment was started at the time when focal PDAC is present, and maintained until signs of illness required euthanasia. In contrast to their effects in the Smad4 wild-type Kras-p53<sup>Lox/+</sup> model, αvβ6 blocking antibodies did not alter the latency or histopathologic features of Smad4 null tumors (Figure 3B). Therefore, genetic inactivation of TGFβ signaling obviates the effect of αvβ6 blockade on tumorigenesis, consistent with a predominant action of the blocking antibodies in inhibiting epithelial TGFβ-Smad4 signaling.

Discussion

Here we show that TGFβ pathway blockade with specific monoclonal antibodies to αvβ6 or TGFβ1-3 accelerated PDAC progression in GEM models. This effect was observed when using antibodies at early and later disease stages, and as a single agent or in combination with gemcitabine. While tumorigenesis was not accelerated in a GEM model lacking Smad4, αvβ6/TGFβ inhibition did not provide any appreciable benefit in this setting. While it is possible that αvβ6/TGFβ pathway inhibition may restrain aspects of malignancy such as metastatic spread, our data indicate that there may be risks in broadly targeting this pathway given its primary function as a tumor suppressor.

Recent findings have supported the ability of GEM models to recapitulate therapeutic responses seen in patients (19). Our experiments illustrate a number of potential advantages of GEM models, such as the capacity to evaluate the impact of an
intervention at different disease stages including pre-invasive disease, and in defined
tumor genotypes. Whereas preclinical studies in xenografts have supported the use of
TGFβ pathway inhibitors in the treatment of PDAC, our work indicates this strategy
carries risk in the context of autochthonous tumors.

It remains possible that there may be contexts in which inhibition of components of
the αvβ6-TGFβ pathway may prove beneficial in PDAC treatment. Both αvβ6 and
TGFβ have been implicated in metastasis, which we were not able to address definitively
in our studies (1/7 controls and 1/15 treated mice exhibited metastasis, which was
insufficient to provide statistical significance). In addition, while direct targeting of αvβ6
or TGFβ may carry risks, it is possible that downstream effectors of the pathway have
strictly tumor-promoting effects, and thus may be effective targets for pharmacologic
blockade. Along these lines a recent study showed TGFβ activates CxC chemokine
signaling in a PDAC GEM model and that inhibition delays tumor progression (20).

Our studies also reveal new insights into the mechanisms of TGFβ activation in
the pancreas and the contributions of this pathway in multi-stage PDAC progression. We
show that global inactivation of TGFβ signaling promotes increased proliferation of the
PanIN epithelial cells and enhances PDAC initiation and progression in a Smad4
dependant manner. Although TGFβ likely has additional functions in regulating the
PDAC microenvironment, these functions do not appear essential for either the tumor
promotion or tumor suppression. We also identify αvβ6 as a critical upstream regulator
of TGFβ signaling in the ductal epithelium and demonstrate that αvβ6 has a previously
unanticipated function in tumor suppression. αvβ6 blockade attenuated Smad2
activation, produced similar biological effects to TGFβ blockade in our mouse models,
and did not affect the progression of Smad4 null tumors. Thus, while αvβ6 has been
shown to promote invasive growth of advanced cancers, our data indicate that the
primary function of αvβ6 in the pancreas is to serve as an upstream component of the TGFβ tumor suppression program. It is worth noting that PDAC in the Kras-Smad4 model arise from cystic precursors rather than PanIN (3, 5), which could also contribute to the differential response to pathway inhibition in this setting.

In summary, this series of experiments highlight the use of GEM cancer models to guide in the clinical development of novel therapeutics and to elucidate signaling pathway circuitry in vivo. We show that αvβ6 and TGFβ act in a common Smad4-dependent PDAC tumor suppressor pathway. Moreover, we conclude that broad use of TGFβ inhibitors in unselected populations of PDAC patients could have detrimental consequences, and in particular, that there is potential for disease acceleration in cancers with an intact TGFβ/SMAD4 signaling pathway.

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References


Figure Legends
**Fig 1. αvβ6 activates TGFβ in the pancreatic epithelium.**

**A)** IHC staining for phospho-Smad2 (top row, 400X) and integrin β6 (bottom row, 200X), during multi-stage progression of the $Kras-p53^{Lox/+}$ PDAC model. Duct (D), acinar (A), islet (I), metaplastic acini (M), and PanIN (P) cells indicated. P-Smad2+ neoplastic and stromal cells designated by yellow and red arrowheads, respectively.

**B)** IHC for integrinβ6 in normal human pancreas, and in multistage PDAC progression.

**C)** *Left:* phospho-Smad2 IHC in PanIN and PDAC from $Kras-p53^{Lox/+}$ mice treated with αvβ6 blocking antibodies or control. *Right:* Phospho-Smad2+ nuclei quantified by automated analysis.

**Fig 2. TGFβ or αvβ6 blockade accelerates PDAC initiation and progression in GEM models.**

**A, B)** Evaluation of the impact of anti-αvβ6 and anti-Tgf-β antibodies on early disease in $Kras-p53^{Lox/+}$ mice. **A)** Schematic indicating the course of PDAC progression in control animals (top), as well as the treatment interval (red line). Mice were euthanized for analysis at 12 weeks. **B)** *Left:* Representative histological images to quantify the proportion diseased pancreas (top) and Ki67 staining to evaluate proliferation of PanIN epithelium (bottom). *Right panel:* Quantification of % of pancreatic area occupied by PanIN and PDAC lesions, treatment groups have significantly increased disease burden compared with controls (*p<0.05). Quantification of Ki67 staining; treatment groups have increased epithelial proliferation compared with controls (**p< 0.005; ***p< 0.001).

**C)** Impact of anti-αvβ6 and anti-Tgf-β antibodies on tumor progression in $Kras-p53^{Lox/+}$ mice. Schematic: treatment was initiated at later stages of disease and continued until clinical signs of illness. *Lower Panels:* Kaplan-Meier analysis. *Left:* Survival is shortened in the anti-αvβ6 (mean 6.6 weeks; p=0.03) or anti-Tgfβ (mean 5.6 weeks; p=0.007)
cohorts compared with isotype control treated animals (mean 8.9 weeks). Survival of untreated animals is shown for comparison (grey line); *p< 0.05. Right: Anti-Tgfβ and anti-αvβ6 antibody treatments reduce survival of gemcitabine (Gem) treated mice.

**Fig. 3. αvβ6 functions through Smad4 tumor suppressor in the pancreas.**

**A)** The relationship between Smad4 and β6 expression was evaluated in Smad4 wildtype and mutant PDAC models. % β6-neoplastic cells in individual tumors (graph on left) and representative IHC images (right panels) are shown. All Smad4 null tumors retain β6 expression (left), whereas subsets of Smad4 wild type tumors either lose (middle) or retain (right) Smad4 expression (*p<0.05). Insets show higher magnification.

**B)** Impact of anti-αvβ6 treatment on PDAC progression in the *Kras-Smad4<sup>Lox/Lox</sup>, Ink4a/Arf<sup>Lox/+</sup> model. Left: Schematic showing experimental design. Right: Kaplan-Meier analysis showing that αvβ6 blockade does not affect survival (n.s.= not significant).
Figure 1

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% phospho-Smad2 + nuclei

- PanIN
- PDAC

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Figure 2

A. PanIN progression study

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Disease Progression

Treatment

Age (weeks)

Treatment starts

Analysis

B. Treatment Group

Control | αvβ6 | Tgfβ

10X H&E

NP

PanIN

PDAC

Disease burden (%)

% Ki67 +

C. PDAC treatment study

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Disease Progression

Treatment

Age (weeks)

Treatment starts

% survival

Weeks of age

Gem/cntrl n=14

Gem/αvβ6 n=9

Gem/Tgfβ n=9

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Figure 3

A  Mouse PDAC IHC

B  Smad4 mutant PDAC treatment study

% αvβ6 negative

Smad4 wt / β6+
Smad4 wt / β6-
Smad4 mut / β6+

Disease Progression

Normal  focal PDAC  advanced PDAC  death

0  4  8  12  16

Treatment starts

% survival

0  50  100

0  5  10  15

cntrl  n=7
αvβ6 n=7

Mouse PDAC IHC

Smad4 wt / β6+
Smad4 wt / β6-
Smad4 mut / β6+

% αvß6 negative

Smad4 wt / β6+
Smad4 wt / β6-
Smad4 mut / β6+

% survival

0  50  100

0  5  10  15

cntrl  n=7
αvβ6 n=7

Disease Progression

Normal  focal PDAC  advanced PDAC  death

0  4  8  12  16

Treatment starts

% survival

0  50  100

0  5  10  15

cntrl  n=7
αvβ6 n=7

Disease Progression

Normal  focal PDAC  advanced PDAC  death

0  4  8  12  16

Treatment starts

% survival

0  50  100

0  5  10  15

cntrl  n=7
αvβ6 n=7

Disease Progression

Normal  focal PDAC  advanced PDAC  death

0  4  8  12  16

Treatment starts

% survival

0  50  100

0  5  10  15

cntrl  n=7
αvβ6 n=7
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